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BMJ Open A systematic review of epigenetic biomarkers in progression from non-dysplastic Barrett's oesophagus to oesophageal adenocarcinoma

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ABSTRACT

Objectives The objective of this systematic review is to identify and summarise studies which examine epigenetic biomarkers in patients with Barrett's oesophagus (BO) and their association with progression to oesophageal adenocarcinoma (OADC). BO is a precursor lesion for OADC. There is no clinical test to predict patients who are likely to progress to OADC. An epigenetic biomarker could predict patients who are at high risk of progression from BO to OADC which could facilitate earlier diagnosis and spare those unlikely to develop cancer from regular invasive surveillance endoscopy.

Setting A systematic search was conducted of the following databases: MEDLINE, MEDLINE in Process, EMBASE, Cochrane Central, ISI Conference Proceedings Citation Index and the British Library's ZETOC. Studies were conducted in secondary and tertiary care settings.

Participants All studies measuring epigenetic change in patients over 18 years old who progressed from non-dysplastic BO to OADC were included. Genetic, in vitro and studies which did not measure progression in the same patient cohort were excluded. Study inclusion and risk of bias of individual eligible studies were assessed in duplicate by two reviewers using a modified Quality in Prognostic Studies tool.

Results 14 studies met the inclusion criteria. 42 epigenetic markers were identified, and 5 studies developed models aiming to predict progression to OADC.

Conclusions The evidence from this systematic review is suggestive of a role for p16 as an epigenetic biomarker for the progression of BO to OADC.

Prospero number CRD42016038654.

INTRODUCTION

Barrett's oesophagus (BO) is defined as an oesophagus in which any portion of the normal distal squamous epithelial lining is replaced by metaplastic columnar epithelium which is clearly visible endoscopically (≥ 1 cm) above the gastro-oesophageal junction and confirmed histopathologically from oesophageal biopsies.¹ BO arises due to long-standing gastro-oesophageal reflux

Strengths and limitations of this study

- Systematic review conducted following strict Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.
- Systematic and reproducible methodology using two independent reviewers.
- All mechanisms of epigenetic change included.
- Limited meta-analysis resulting from lack of standardisation between studies.
- Small patient numbers in the included studies.

disease (GORD) and chronic inflammation and is a precursor lesion for oesophageal adenocarcinoma (OADC) with progression through the metaplasia-dysplasia-carcinoma sequence.² The likelihood of developing OADC is increased 1.7 times in patients with GORD, increasing to 10.6 times with BO.³ The incidence of OADC has risen in parallel with increasing obesity and GORD in Western populations.⁴ Patients with OADC who are diagnosed at an early disease stage benefit from much improved 5-year survival rates of up to 39%⁵ in comparison to less than 13% with invasive late stage lesions,⁶ highlighting the importance of early diagnosis and treatment.

Currently, there is no robust way of predicting which patients with BO will progress to OADC. Clinical and histological information is currently the only tools at the clinician's disposal to aid early detection of OADC. The British Society of Gastroenterology recommends endoscopic surveillance of patients with BO, and the American College of Gastroenterology endorses screening of high-risk patients for BO.^{1 7} Endoscopic surveillance is invasive and expensive, and despite rigorous biopsy protocols, dysplasia and early cancers can be missed. A recent meta-analysis published in 2012 demonstrated lower risk for progression

of non-dysplastic BO (NDBO) to OADC than previously reported with a pooled 0.33% (95% CI 0.28% to 0.38%) annual incidence of OADC in patients with NDBO.⁸ The annual incidence rate of OADC for patients with BO with high-grade dysplasia (HGD) is 7%–19%.^{9–11}

Epigenetics is an emerging field which describes mechanisms of alteration of gene regulation and expression without changing the genetic code.¹² The most widely recognised mechanisms of epigenetic change are covalent modifications and altered gene expression by non-coding RNAs. Covalent modifications alter the structure of DNA and include DNA methylation and histone modification.¹² Mechanisms of epigenetic change are discussed in more detail in the protocol for this systematic review.¹³

Epigenetic changes in ulcerative colitis (UC) are well described^{14–17} and have been shown to occur before neoplasia occurs at an early stage of UC-associated carcinogenesis.¹⁸ UC-associated carcinomas progress in a similar fashion to OADC as a result of chronic inflammation through the metaplasia-dysplasia-adenocarcinoma sequence.¹⁹ The Enhanced Neoplasia Detection and Cancer Prevention in Chronic Colitis trial is investigating whether a panel of methylated biomarkers detected in endoscopic biopsy samples can be used as a tool in conjunction with screening colonoscopy to help risk stratify patients who are at higher risk of progressing to carcinoma.²⁰ In light of this, there is a need to consolidate the literature on epigenetic changes in Barrett's carcinogenesis to determine if such changes provide a method of risk stratifying patients who are at risk of progression to OADC.

A scoping search was performed using MEDLINE, the Cochrane Library and internet sources to identify any systematic reviews or meta-analyses on epigenetic biomarkers in BO and oesophageal cancer (OC). Nine systematic reviews and meta-analyses were identified^{21–29} which included mixed patient populations with OADC and oesophageal squamous cell carcinoma with only three reviews incorporating patients with BO.^{24 25 29} Seven reviews concentrated on a single type of epigenetic alteration with four investigating DNA methylation^{21–23} and three looking at micro RNA (miRNA) expression.^{25–27} The remaining two reviews investigated genetic alterations in progression of BO to OADC.^{28 29} No systematic reviews drawing together all aspects of epigenetic change within the field of Barrett's carcinogenesis were identified.

AIM

To identify and summarise studies which examine epigenetic biomarkers in patients with BO and their association with progression to OADC.

METHODS

Details of the methodology were registered on PROSPERO (CRD42016038654) and have also been published.¹³ A summary is reported here.

Patient and public involvement

This research question was developed to address the issue of BO surveillance. The priority for patients is early diagnosis of OADC and accurate surveillance. In order to achieve this, it is imperative that the correct patient group, that is, those who are at highest risk of progression to OADC, is placed under the most intensive of surveillance, and those at a lower risk can be spared such frequent invasive investigation. Epigenetic biomarkers may provide a robust way of risk stratifying patients for BO surveillance.

Patients and the public were not involved in the development of this systematic review.

Eligibility criteria

Any prospective and retrospective primary studies were eligible for inclusion provided they measured epigenetic markers in patients over the age of 18 years with BO. To be included, the study must have reported on progression from NDBO to BO with HGD or OADC in the same patient cohort. Relevant epigenetic markers are DNA methylation, histone modification, chromatin remodelling and micro and non-coding RNAs. Studies were excluded if they were case reports, narrative reviews, in vitro studies (eg, using cell lines), studies of genetic (rather than epigenetic) mutations, studies using biomarkers to predict a response to treatment (eg, chemotherapy) or animal studies.

Search

A systematic search of the literature to the end of February 2018 was undertaken. Text and index terms relating to the population (BO), the prognostic marker (epigenetic change) and the outcome (BO with HGD or OADC) were combined (see online supplementary appendix 1 for sample search strategy in MEDLINE). No study design, date or language restrictions were applied. MEDLINE, MEDLINE in Process, EMBASE, Cochrane Central, ISI Conference Proceedings Citation Index and the British Library's ZETOC were searched from inception. Reference lists of identified studies and systematic reviews were screened for any additional relevant primary studies. Registers of clinical trials (ClinicalTrials.gov and ICTRP) were searched for ongoing studies.

Study selection

Two reviewers (TN and CLT) independently screened all titles and abstracts to identify potentially relevant studies using prespecified screening criteria. Full texts of potentially relevant articles were assessed against prespecified eligibility criteria. Eligibility was determined by two reviewers (TN and CLT) independently with any discrepancies resolved by discussion or referral to a third reviewer (OT).

Data extraction

Data extraction of the included studies was carried out by one reviewer (TN) using a standardised data extraction form and checked independently by a second reviewer

(OT). Discrepancies were resolved by discussion or referral to a third reviewer (CLT and JD). Data were extracted on study design characteristics, patient characteristics, prognostic marker and outcomes.

Quality assessment

The risk of bias of individual eligible studies was assessed in duplicate by two reviewers (TN and OT) using a modified Quality in Prognostic Studies tool.³⁰ Risk of bias criteria was related to study participation (eg, method of sampling), study attrition, prognostic factor measurement and selection (eg, reliability of epigenetic technique and publication bias), outcome assessment (eg, undertaken in duplicate) and study confounding factors (measured and adjusted for). The main confounders are considered to be age, obesity, smoking and alcohol intake.¹ Selected elements from prediction study risk of bias assessment tool (PROBAST) which is currently under development (Wolff R) were used to assess the methodological quality of prognostic models in a similar fashion to Ensor *et al*'s 2016 systematic review of prognostic models of venous thromboembolism³¹ including aspects on patient selection, statistical models used and model validation.

Synthesis

Synthesis was narrative, with main findings tabulated. Studies were grouped by individual epigenetic marker

or panel of markers. A lack of consistency in reported outcome metrics and heterogeneity relating to study design, length of follow-up, frequency of endoscopy and biopsy and experimental technique precluded any quantitative synthesis. Most studies presented results as percentage methylation or a ratio of differential methylation. Formal assessment of publication bias was not possible.

Reporting

Reporting of this systematic review was according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses³² guidelines (online supplementary appendix 2)

SYSTEMATIC REVIEW RESULTS

Overall, 3995 records were screened, and 14 studies met the inclusion criteria (see figure 1 for study selection process and reasons for exclusion). The most common reasons for exclusion were lack of progression in the same population from NDBO to HGD or OADC, in vitro experimentation or no epigenetic change analysed in patient samples.

Study characteristics

Table 1 shows the main study characteristics. All included studies were of a similar retrospective cohort design. In

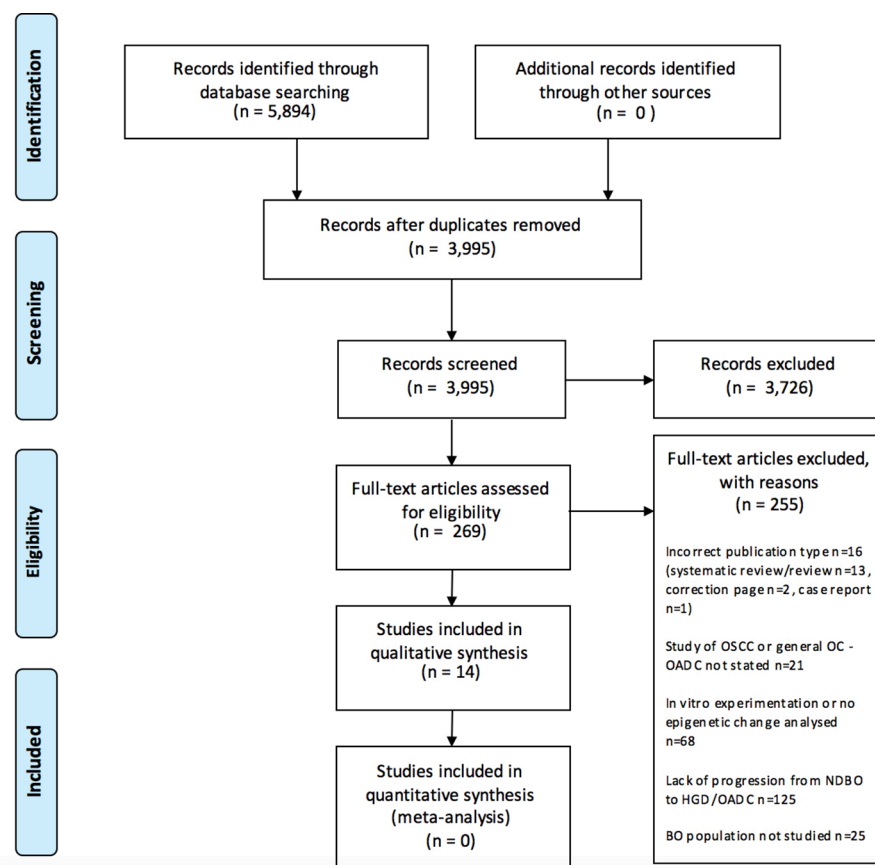


Figure 1 PRISMA 2009 flow diagram with reasons for exclusion. BO, Barrett's oesophagus; HGD, high-grade dysplasia; NDBO, non-dysplastic Barrett's oesophagus; OADC, oesophageal adenocarcinoma; OC, oesophageal cancer; OSCC, oesophageal squamous cell carcinoma; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

Table 1 Summary table of included studies

Author/year	Study type	Study population	Patient no (P:NP)	Patient demographics (mean age, M:F ratio)	Epigenetic analysis	Markers analysed	Result
Agarwal <i>et al</i> 2012 ⁴⁶	Retrospective cohort	Pathology archives at Baltimore Veteran's Affairs Medical Centre and Johns Hopkins University School of Medicine	9 (5:4)	Not stated	CpG methylation (244K human CpG microarray)	Extragenic x 3 Intra_BCL11B Intra_CCDC57 Intra_F10 Pro_CKB Pro_ELAVL3-ZNF653 Pro_GPR177 Pro_HOXB7 Pro_IGF1R Pro_ITGB8 Pro_JARID 1B Pro_JUND Pro_LAMA5 Pro_LOC55565 Pro_MGC35308 Pro_MMD2 Pro_TAF10 Pro_TLX3 Pro_UBP1 Pro_WNK4 Pro_WWC1 Pro_ZBTB7B Pro_ZNF358	0.45—relative CpG methylation 0.17 0.23 1.86 0.83 0.6 0.7 0.67 0.58 0.55 0.35 0.54 0.45 0.76 0.52 1.34 0.43 0.31 0.71 0.76 0.68 0.56 1.25
Barrett <i>et al</i> 1999 ³⁷	Retrospective cohort	Fred Hutchinson Cancer Research Centre+University of Washington	31 (25:6)	Not stated	CpG methylation (methylation-specific PCR)	CDKN2A	Hypermethylation in premalignant samples
Boerwinkel <i>et al</i> 2014 ⁴³	Retrospective cohort	AMC BO Surveillance	44 (48:10)	Mean age=6632M:12F	CpG methylation (methylight methylation-specific PCR)	HPP1 p16 RUNX3	NS P hypermethylated NS
Clément <i>et al</i> 2006 ³⁵	Retrospective cohort	Lausanne, Switzerland	28* (12:16)	Not stated	CpG methylation (methylation-specific PCR)	APC CDKN2A SFRP1 TIMP3 TERT	P hypermethylated NS Hypermethylated in all samples P and NP P hypermethylated P hypermethylated
Clément <i>et al</i> 2008 ³⁴	Retrospective cohort	Lausanne, Switzerland	31 (16:15)	Not stated	CpG methylation (methylation-specific PCR)	WIF-1	P hypermethylated

Continued

Table 1 Continued

Author/year	Study type	Study population	Patient no (P:NP)	Patient demographics (mean age, M:F ratio)	Epigenetic analysis	Markers analysed	Result
Eads <i>et al</i> 2001 ⁴⁴	Retrospective cohort	Norris Comprehensive Cancer Centre	20 (12:8)	Not stated	CpG methylation (methylight methylation-specific PCR)	CALCA CDKN2A ESR1 MGMT MYOD1 TIMP3	P hypermethylated P hypermethylated P hypermethylated P hypermethylated P hypermethylated P hypermethylated
Jin <i>et al</i> 2009 ⁴¹	Case control	Five participating US clinics	195* (50:145)	P significantly older than NP	CpG methylation (methylation-specific PCR)	HPP1 p16 RUNX3 CDH13 TAC1 NELL1 AKAP12 SST	P hypermethylated P hypermethylated P hypermethylated NS NS NS NS NS
Klump <i>et al</i> 1998 ⁴⁵	Retrospective cohort	Under routine Barrett's surveillance	14 (10:4)	P mean age 57.48M:2F	CpG methylation (methylation-specific PCR)	p16	P hypermethylated
Moinova <i>et al</i> 2012 ⁴²	Retrospective cohort	Retrospective collection from pathology database	5 (2:3)	Not stated	CpG methylation (methylation-specific PCR)	VIM	VIM methylation present in NDBO, not relevant in progression
Puertas Canteria 2012 ⁴³	Retrospective cohort	No details	55 (6:14)	Not stated	CpG methylation (bisulfite pyrosequencing)	p16	Methylation P 12.04% versus NP 6.53%
Revilla-Nuin <i>et al</i> 2013 ⁴³	Retrospective cohort	1982 trial assessing medical versus surgical therapy for prevention of progression of BO. Randomly selected	5 (7:17)	Not stated	miRNA expression analysis	miR-192 miR-194 miR-196a miR-196b	Overexpressed AUROC 0.61 Overexpressed AUROC 0.70 Overexpressed AUROC 0.80 Overexpressed AUROC 0.74
Sato <i>et al</i> 2008 ⁴⁰	Retrospective cohort	MAYO and UMD mixed cohort	62 (28:34)	Not stated	CpG methylation (methylation-specific PCR)	HPP1 p16 RUNX3	P hypermethylated P hypermethylated P hypermethylated
Schulmann <i>et al</i> 2005 ³⁹	Retrospective cohort	Baltimore Veteran's Affairs hospitals and University of Maryland Hospitals	53 (8:45)	P mean age 63.34M:0F; segment length 10.0 cm NP mean age 62.235M:3F; segment length 5.8 cm	CpG methylation (methylation-specific PCR)	RUNX3 HPP1 p16 CRBP1 TIMP3 APC	P hypermethylated P hypermethylated P hypermethylated Hypermethylation not independently associated with P Hypermethylation not independently associated with P Hypermethylation not independently associated with P
Wang <i>et al</i> 2009 ³⁸	Retrospective cohort	Johns Hopkins under surveillance for GORD/BO	57 (7:50)	No significant differences in N versus NP; Data not provided	CpG methylation (methylation-specific PCR)	APC p16	P hypermethylated P hypermethylated

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; BO, Barrett's Oesophagus; GORD, gastro-oesophageal reflux disease; M:F, male-to-female ratio; miRNA, micro RNA; NDBO, non-dysplastic BO; NP, non-progressing patients; NS, not statistically significant; P, progressing patients; PLSD, protected least significant difference; ROC, receiver operating characteristic.

Table 2 Models aiming to predict progression to OADC

Author/year	Study type	Patient no (P:NP)	Model used	Result
Clément <i>et al</i> 2006 ³⁵	Retrospective cohort	28* (12:16)	APC+TIMP3+TERT	Hypermethylation in P versus NP (P 81% vs NP 26% P<0.0001)
Jin <i>et al</i> 2009 ⁴¹	Retrospective cohort	195* (50:145)	Biomarker panel (p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12, SST)	AUROC 0.72
			Biomarker panel+age (p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12, SST)	AUROC 0.85
Sato <i>et al</i> 2008 ⁴⁰	Retrospective cohort	62 (28:34)	Methylation index (p16, HPP1, RUNX3)	Hypermethylation in P versus NP AUROC 0.75 (no CI stated)
			Methylation index (p16, HPP1, RUNX3), segment length, pathology	AUROC 0.79 (95% CI 0.6968 to 0.8853) Sensitivity 91.4 Specificity 51.8
Schulmann <i>et al</i> 2005 ³⁹	Retrospective cohort	53 (8:45)	Age, segment length, HPP1, TIMP3, APC, p16, CRBP1, RUNX3	HPP1, p16, RUNX3 independent risk factors in multivariate analyses Model combined HR index >5 leads to an increased likelihood of progression within 2 years
Wang <i>et al</i> 2009 ³⁸	Retrospective cohort	57 (7:50)	P16+APC	Hypermethylation of both APC and p16 OR 14.97 (95% CI 1.73 to ∞, P=0.012) for neoplastic progression

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; NP, non-progressing patients; OADC, oesophageal adenocarcinoma; P, progressing patients.

total, 12 of 14 studies reported patient numbers. A total of 404 patients were included. Two studies reported total lesions only (n=223). CpG promoter methylation was investigated in 13 studies and miRNA expression in one study.³³ Forty-two unique epigenetic markers were reported. No studies investigated histone modification. Thirteen included studies analysed 38 differentially methylated CpG promoter sites, nine used methylation-specific PCR,^{34–42} two used methylight methylation-specific PCR,^{43–44} one used bisulfite pyrosequencing⁴⁵ and one used methylation microarray techniques.⁴⁶

Prognostic models

Five studies developed models aiming to predict progression to OADC (table 2).^{35–38–41} Three models included both epigenetic markers and clinical parameters.^{39–41}

Schulmann *et al*³⁹ performed a retrospective longitudinal analysis of 53 patients contributing 106 specimens enrolled in a BO surveillance programme using Cox proportional hazards regression. Initially, 10 candidate prognostic marker genes were assessed using cross-sectional data. Six of the genes (HPP1, TIMP3, APC, p16, CRBP1 and RUNX3) which demonstrated hypermethylation in patients with OADC and relative hypomethylation in normal oesophageal tissues were investigated in the longitudinal study. All except APC were associated with progression (HGD and OADC combined) in univariate analysis. A multivariate model including all six genes, age and segment length found evidence that HPP1, p16

and RUNX3 were independently prognostic. The performance of the full model (all eight covariates) was assessed by calculating the exponentiated multiplier of baseline hazard for covariates for each specimen (HR index). For specimens taken within 2 years of progression, the HR index was >5 in progressors compared with <5 in non-progressors. The authors report that the HR index was not predictive for specimens taken over 2 years before progression. The study reports OR but doesn't explain how as the Cox model produces HRs. The authors note that the sample size is small, the study is retrospective, other potential clinical predictors were unavailable and the model uses a combined end point of HGD and OADC. In univariate analysis, TIMP3 had an OR for progression of 1.68 (95% CI 1.14 to 2.38) but this flipped to 0.5 (95% CI 0.22 to 1.04) in the multivariate analysis. The authors conclude that further validation of the markers (HPP1, p16 and RUNX3), ideally in prospective multicentre trials, is required.

Sato *et al*⁴⁰ performed a retrospective cohort study on 62 patients providing a total of 118 specimens. They developed a model using linear discriminant analysis incorporating both clinical and epigenetic markers. Sex, BO segment length and histological diagnosis were combined with p16, HPP1 and RUNX3 CpG methylation status as well as a methylation index with a score of 0–3 was considered. The best receiver operating characteristic (ROC) curve for a 4-year follow-up in this cohort was

generated using segment length, pathology and methylation index with an area under ROC (AUROC) of 0.7910 (95% CI 0.6968 to 0.8853) with a specificity and sensitivity of 91.4% and 51.8%, respectively. Change in AUROC between this model and a model including predictive markers alone was not assessed. Patient selection criteria were not reported, and the small sample size (62 patients), the large number of possible parameter combinations ($n=127$), and the cut-points for variables were chosen to optimise performance in this dataset results in a high level of uncertainty about the optimal parameter set. No external validation was performed.

Jin *et al*⁴¹ performed a double-blind multicentre case-control study of 195 tissue specimens using an eight methylation biomarker panel of p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12 and SST combined with patient age to predict which patients will progress to OADC. When assessed individually, p16, HPP1 and RUNX3 were associated with progression to OADC ($P<0.05$). When the panel of all eight markers was used compared with age alone as a predictor of progression, the increment in AUROC was 0.114 in a 4-year follow-up (0.630 age alone and 0.753 age+markers), demonstrating a clinically important improvement in the predictive power of the model within the dataset. This study compared clinical factors between progressor and non-progressor groups and found no significant difference in gender, body mass index (BMI), BO segment length, smoking status or alcohol consumption. External validation was not performed.

Clément *et al*³⁵ performed a retrospective cohort study of 28 tissue specimens. Eighty-one per cent of patients ($n=12$) with combined hypermethylation of APC, TIMP3 and TERT progressed to OADC compared with 26% of non-progressors ($n=16$) ($P<0.0001$). It was suggested that in combination, these three markers could be used to predict which patients are at higher risk of progression. No sensitivities or specificities were reported, and there was no ROC analysis. There is no indication of independent prognostic value, and no internal or external validation was performed using this model.

Wang *et al*³⁸ performed a retrospective cohort study on 7 progressor and 50 non-progressor patients. They reported that hypermethylation in both p16 and APC was a strong predictor of progression to dysplastic BO or OADC. Patients who were negative for both p16 and APC hypermethylation did not progress. Hypermethylation of both APC and p16 yielded an OR of 14.97 (95% CI 1.73 to ∞ , $P=0.012$) for subsequent progression to HGD or OADC. A limitation of this study is the short follow-up time of 4.1 years for the non-progressor group which may not be sufficient time for dysplasia or neoplasia to develop. The reported CI is extremely wide, making it difficult to accurately interpret the OR.

Individual markers analysed

Forty-two individual epigenetic markers were analysed in the 14 included studies. Ten studies investigated one

or more of the following five individual markers: p16 (CDKN2A), RUNX3, TIMP3, HPP1 and APC. Details of markers, assessment methods and findings can be found in tables 3–7. All five markers demonstrated CpG hypermethylation in patients who progressed from NDBO to OADC in at least one of the included studies. *p16* (a tumour suppressor protein encoded by the *CDKN2A* gene) offered the most experimental data with 10 studies reporting on 220 progressor samples compared with 332 non-progressor samples. Eight of the 10 studies demonstrated a statistically significant difference in hypermethylation at p16.^{36 38–41 43–45} Barrett *et al*³⁷ demonstrated CpG hypermethylation of CDKN2A in seven progressor samples of a specific genetic clonality; however, no control group was analysed in parallel for this specific marker in this study.

RUNX3 and *HPP1* were analysed in four studies^{39–41 43} as individual markers. Overall, 134 progressors and 234 non-progressors were studied. Three studies^{39–41} demonstrated statistically significant hypermethylation in both RUNX3 and HPP1 CpG sites in progressor samples; however, one study⁴³ found no difference for either HPP1 or RUNX3.

Three studies investigated *TIMP3* CpG methylation^{35 39 44} (32 progressors and 69 non-progressors). One study showed a statistically significant difference in CpG hypermethylation between progressors and non-progressors.³⁵ One study showed significant hypermethylation and calculated an OR of 1.68 (95% CI 1.14 to 2.38) for progression on univariate analysis³⁹; however, TIMP3 could not be regarded as an independent risk factor as the OR dropped to 0.5 (95% CI 0.22 to 1.04) on multivariate analysis. The final study⁴⁴ showed significantly increased TIMP3 methylation in non-dysplastic tissues of patients with associated dysplasia elsewhere in the oesophagus; however, there was no difference between progressors and non-progressors.

Three studies analysed *APC*^{35 38 39} (26 progressors and 116 non-progressors). Wang *et al*³⁸ demonstrated hypermethylation in progressors. Clément *et al*³⁵ reported hypermethylation but did not provide any statistical analyses. Wang *et al* calculated an OR of 9 (95% CI 1.01 to 88.52) for hypermethylation of APC in progressors. Conversely, Schulmann *et al*³⁹ found no evidence of a difference (OR=1.00 (95% CI 0.59 to 1.33)) in progression with or without hypermethylation of APC. All three studies have similar sample size, study design and experimental techniques. These contradictory findings with limited statistical validation and wide CIs call into question the validity of APC as an epigenetic marker of BO progression.

The main methodological limitations across the studies were poor reporting of patient characteristics and study populations, including patient selection. There was little information about loss to follow-up, and it was often unclear exactly which samples were used and at which time-points in longitudinal analyses. There was limited blinding of researchers to the histology, and progressor status of the

Table 3 P16/CDKN2A

Author/year	Patient no (P:NP)	Experimental technique used	Methylation threshold definition	Statistical analysis	Result
Barrett <i>et al</i> 1999 ³⁷	49 (39:6)	Methylation-specific PCR	Positive or negative PCR on agarose gel	Not stated	Seven progressor patients with specific genetic abnormality (LOH at 17p and 9p) displayed hypermethylation at CDKN2A. No numerical value offered
Boerwinkel <i>et al</i> 2014 ⁴³	44 (48:10)	Methylight methylation-specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation: p16 cut-off=0.02	Mann-Whitney test	Raw MSP values showed Hypermethylated in P P<0.05
Clément <i>et al</i> 2006 ³⁵	28* (12:16)	Methylation-specific PCR	Intensity of methylation-specific dot-blot assay compared	Not stated	0% P versus 12% NP methylated No significant difference between P and NP
Eads <i>et al</i> 2001 ⁴⁴	20 (12:8)	Methylight methylation-specific PCR	Intensity of methylated genes compared with controls. 'PMR' value of 4 used as cut-off to indicate methylated gene	Fisher's PLSD	Intensity of PCR band used for quantitative analysis Hypermethylated in P P=0.0048
Jin <i>et al</i> 2009 ⁴¹	195* (50:145)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Student's t test and chi-squared test AUROC	NMVs P:NP 0.138:0.069 AUROC=0.628 (0.534, 0.722) 90% sensitivity 90% specificity Hypermethylated in P P=0.0066
Klump <i>et al</i> 1998 ³⁶	14 (10:4)	Methylation-specific PCR	Yes/no detection of PCR product	Chi-squared test	Hypermethylated in P 8% P versus 0% NP P=0.0001
Puertas Canteria 2012 ⁴⁵	35 (6:14)	Bisulfite pyrosequencing	Quantitative CpG methylation technique	No statistical analysis	Hypermethylated in P Methylation grade 12.04% P versus 6.53% NP
Sato <i>et al</i> 2008 ⁴⁰	62 (28:34)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	LDA, LOOCV, AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index=0.0335 90% sensitivity 90% specificity P=0.00576
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P OR 1.74 P=0.0005
Wang <i>et al</i> 2009 ³⁸	57 (7:50)	Methylation-specific PCR	No threshold described	OR using univariate logistic regression	Hypermethylated in P OR 10.02 P=0.034

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; LDA, linear discriminant analysis; LOH, Loss of Heterozygosity; LOOCV, leave-one-out cross-validation; MSP, methylation specific PCR; NMV, normalised methylation value; NP, non-progressing patients; P, progressing patients; PLSD, protected least significant difference; PMR, percent methylated reference.

Table 4 RUNX3

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Boerwinkel <i>et al</i> 2014 ⁴³	44 (48:10)	Methylight methylation-specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation. RUNX3 cut-off=0.02	Mann-Whitney test	Raw MSP values compared but no numerical value offered P versus NP not significantly differentially methylated
Jin <i>et al</i> 2009 ⁴¹	195* (50:145)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Student's t test and chi-squared test AUROC	Hypermethylated in P NMV 0.104:0.063 AUROC=0.671 (0.586, 0.756) 90% sensitivity 90% specificity P=0.0002
Sato <i>et al</i> 2008 ⁴⁰	62 (28:34)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	LDA, LOOCV, AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index=0.0216 90% sensitivity 90% specificity P=0.016
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P OR=1.80 P=0.0267

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; LDA, linear discriminant analysis; LOOCV, leave-one-out cross-validation; MSP, methylation specific PCR; NMV, normalised methylation value; NP, non-progressing patients; P, progressing patients.

samples was often not described. Confounding factors were described in six of the studies,^{33 38–41 46} but only Sato *et al*⁴⁰ adjusted for BO segment length and patient sex in their predictive model. Full details of quality assessment can be found in table 8.

Given these methodological uncertainties, the findings need to be viewed with caution.

DISCUSSION

This systematic review of the literature of epigenetic markers and their role in predicting progression of BO to HGD and OADC has revealed a heterogeneous and disparate dataset. Fourteen studies were identified, with five incorporating prognostic models. This is the first systematic review to examine all evidence on epigenetic change and its role in Barrett's carcinogenesis. It suggests a role for p16 hypermethylation as an individual epigenetic biomarker in predicting progression from BO to OADC.^{35–41 43–45} However, a paucity of evidence for other epigenetic markers (and combinations), poor reporting of patient characteristics and methods employed and lack of external model validation limit the conclusions that can be drawn.

Only 14 studies were identified with small patient numbers (median 31 (5–195)). The extent of loss to follow-up was usually poorly described. All studies suffered from a paucity of clinical information and a

lack of reporting of study patient demographics. Those studies which did report comorbidities and potential confounding factors such as BMI, smoking status, age, histological diagnosis and BO segment length rarely adjusted for these in their analysis. Without adjusting for these factors, it is difficult to assess the incremental predictive clinical value of any epigenetic changes. Future models should explore the predictive ability of epigenetic changes in the context of clinical variables. In their study, Riley *et al*⁴⁷ concluded that provision of individual patient data could have overcome the majority of the reporting issues including poorly reported summary statistics, adjustment factors and outcome measures used.

It is challenging to compare data from different studies due to variable measurement of epigenetic change. Five different experimental techniques were used: miRNA analysis (n=1)³³ and CpG methylation including, methylation-specific PCR (n=9),⁴⁸ methylight methylation-specific PCR (n=2),⁴⁹ bisulfite pyrosequencing (n=1)^{45 50} and methylation microarray technology (n=1).⁴⁶ Early methods of measuring differential DNA methylation involve semiquantitative reporting of results including analysis of intensity of PCR product bands on agarose gel. More advanced techniques use computerised photometry, although differing PCR conditions and equipment may result in variability

Table 5 HPP1

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Boerwinkel <i>et al</i> 2014 ⁴³	44 (48:10)	Methylight methylation-specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation. HPP1 cut-off=0.05	Mann-Whitney test	P versus NP not significantly differentially methylated
Jin <i>et al</i> 2009 ⁴¹	195* (50:145)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Student's t test and chi-squared test AUROC	Hypermethylated in P AUROC=0.647 (0.556, 0.739) P=0.0025
Sato <i>et al</i> 2008 ⁴⁰	62 (28:34)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	LDA, LOOCV, AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index 0.028 90% sensitivity 90% specificity P=0.018
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P OR=1.77 P=0.0311

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; LDA, linear discriminant analysis; LOOCV, leave-one-out cross-validation; MSP, Methylation Specific PCR; NP, non-progressing patients; P, progressing patients.

between laboratories. More advanced techniques such as bisulfite pyrosequencing and methylation microarrays can provide an accurate methylation percentage at individual CpG sites which are suitable for quantitative analysis. The 12 studies using methylation-specific PCR revealed a global pattern of hypermethylation of progressor patients in the CpG regions of interest^{34–44};

however, each used differing methylation thresholds and outcome measures for reporting positive results. One study⁴⁵ used bisulfite pyrosequencing to analyse p16, but no further markers were analysed on more than one platform. The different methods used, as well as the different outcome metrics reported and lack of clearly reported clinical information, meant that there

Table 6 TIMP3

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Clément <i>et al</i> 2006 ³⁵	28* (12:16)	Methylation-specific PCR	Intensity of methylation-specific dot-blot assay compared	Not stated	Hypermethylated in P % samples methylated P=91%: NP=23% P<0.0001
Eads <i>et al</i> 2001 ⁴⁴	20 (12:8)	Methylight methylation-specific PCR	Intensity of methylated genes compared with controls. 'PMR' value of 4 used as cut-off to indicate methylated gene	Fisher's PLSD	Hypermethylated in non-dysplastic tissue in patients with associated dysplasia; however, not statistically significant when comparing P versus NP P=0.13
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P, but not independent risk factor in multivariate analysis OR=1.68 univariate OR=0.50 multivariate P=0.0109

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). NP, non-progressing patients; P, progressing patients; PLSD, protected least significant difference; PMR, percent methylated reference.

Table 7 APC

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Clément <i>et al</i> 2006 ³⁵	28* (12:16)	Methylation-specific PCR	Intensity of methylation-specific dot-blot assay compared	No statistical analysis offered	Hypermethylated in P methylation: P=100% versus NP=36%
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylation not an independent risk factor for P OR=1.00 (95% CI 0.59 to 1.33) P=0.99
Wang <i>et al</i> 2009 ³⁸	57 (7:50)	Methylation-specific PCR	No threshold described	OR using univariate logistic regression	Hypermethylated in P methylation: P=86% versus NP=40% P=0.02 OR=9 (1.01–80.52) P=0.049

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). NP, non-progressing patients; P, progressing patients.

was insufficient clinical and methodological homogeneity. Meta-analysis was, thus, not appropriate, despite individual epigenetic markers being reported in up to 10 independent studies.

Five models looked at various combinations of epigenetic markers and clinical risk factors. Findings from four of the models suggest a potential role for p16 in combination with HPPI1, RUNX3 and/or APC and clinical factors of segment length or age. All models used slightly different combinations of model parameters and reported analyses differently, thus making it difficult to compare findings between models. All suffered from a lack of external validation, and there were uncertainties around the representativeness of the included populations. More research is needed to validate and further explore these initial findings.

P16, a tumour suppressor protein, is the most consistently hypermethylated CpG site in progressors. Hypermethylation leads to reduced p16 production, allowing neoplastic cells to progress from G1 to S phase of cell cycle and replicate uncontrollably. p16 deletions are observed in melanoma, oesophageal, lung, pancreatic, mesothelioma, bladder, head and neck squamous cell carcinoma, breast, lymphocyte, brain, ovarian, osteosarcoma and renal cancer cell lines.^{51 52} In BO, p16 genetic mutations have been shown to occur early in tumorigenesis and may appear before dysplasia has occurred.⁵³ Timmer *et al*⁵⁴ demonstrated the presence of p16, MYC and aneusomy in a model with age and segment length determined whether patients were at low or high risk of progression from NDBO to OADC. Without further in-depth temporal analysis, it remains unclear whether the index abnormality of Barrett's carcinogenesis is epigenetic or genetic in origin.

The pattern of hypermethylation in RUNX3 and HPPI1 in progressors was less clear with not all studies demonstrating a statistically significant difference between progressor and non-progressor patients. TIMP3 and APC provide variable results in the literature, and much

smaller patient groups are analysed. Agarwal *et al* reported a predominance of hypomethylation in progressors (16 of 19 CpG sites) with only three CpG sites (Pro_MMD2, Pro_ZNF358 and Intra_F10) hypermethylated. The methylation microarray used in this study can analyse 28 700 unique CpG sites, whereas the latest technology offers up to 850 000 sites on a single chip.⁵⁵ With only a single study using an outdated technique, it is not possible to say whether CpG hypomethylation is a driver in progressive BO. Prior to this technology being available, researchers had to carefully select CpG sites of interest for analysis; selection was based on previous research and literature review giving rise to a positive selection and publication bias. This is a well-recognised phenomenon in prognostic factor research. Sekula *et al*⁵⁶ reviewed published and unpublished work into p53's role in bladder cancer and discovered that 31% of observational studies were unpublished in a 15-year period. While Sekula's review is concerned with a different pathology, the type of included studies is of a similar design to those included in this review. A combination of positive publication bias and marker selection bias raises questions as to the validity of the selected markers in studies demonstrating CpG hypermethylation. Selective reporting may also erroneously inflate the importance of individual prognostic markers. Kyzas *et al*⁵⁷ analysed published and unpublished data sought directly from researchers investigating the importance of TP53's role in head and neck squamous cell carcinoma on patient mortality. They found that if all published and unpublished data were included in their meta-analysis, the risk ratio decreased from 1.38 to 1.16 (95% CI 0.99 to 1.35 P=0.06). They also had difficulty conducting their analysis due to non-standardised definitions and reporting. Heterogeneity in definitions and reporting was also identified within our dataset, and it must also be considered whether selective reporting of results is over stating the importance of individual markers such as p16 in the progression of BO. Similarly, if ever increasing numbers of CpG sites

Table 8 Quality assessment for prognostic factor studies including prognostic models

Study	Representative patient sample?	Loss to follow-up equal in both arms?	Study conducted prospectively (PRO) or retrospectively (RET)?	Samples collected prospectively (PRO) or retrospectively (RET)?	Sampling method: quadrantic biopsy used?	Validated epigenetic technique used?	Epigenetic analysis undertaken blindly? (without knowledge of histology)	Epigenetic analysis undertaken in duplicate?	Was the threshold defined?	Histology undertaken in duplicate?	Histology undertaken blindly? (without knowledge of epigenetic finding)	Were confounding factors described? (eg, obesity, age, smoking, alcohol)	Were confounding factors adjusted for analysis?	Which confounding factors were adjusted for?
Agarwal <i>et al</i> 2012 ⁴⁶	Unclear	Unclear	PRO	RET	No	Unclear	Yes	Unclear	Yes	Unclear	No	Yes	No	N/A
Barrett <i>et al</i> 1999 ³⁷	Yes	Unclear	PRO	RET	No	Snap frozen	Yes	Unclear	Unclear	Unclear	Unclear	No	No	N/A
Boerwinkel <i>et al</i> 2014 ⁴³	Yes	Unclear	PRO	RET	Yes	FFPE	Yes	Unclear	Yes	Yes	Yes	No	No	N/A
Clément <i>et al</i> 2006 ³⁵	Unclear	Unclear	PRO	RET	No	FFPE	Yes	Unclear	Unclear	Unclear	Unclear	No	No	N/A
Clément <i>et al</i> 2008 ³⁴	Unclear	Unclear	PRO	RET	No	FFPE	Yes	Unclear	Unclear	Unclear	Unclear	No	No	N/A
Eads <i>et al</i> 2001 ⁴⁴	Unclear	Unclear	PRO	RET	No	Snap frozen	Yes	Unclear	Yes	Unclear	Unclear	No	No	N/A
Jin <i>et al</i> 2009 ⁴¹	Unclear	Unclear	RET	PRO	Yes	Snap frozen	Yes	Unclear	Yes	No	Yes	Yes	No	N/A
Klump <i>et al</i> 1998 ³⁶	Unclear	Yes	RET	RET	No	FFPE	Yes	No	Yes	Unclear	No	No	No	N/A
Moinova <i>et al</i> 2012 ⁴²	Unclear	Unclear	Unclear	Unclear	No	FFPE	Yes	No	Yes	Unclear	Unclear	No	No	N/A
Revilla-Nuin <i>et al</i> 2013 ³³	Yes	Unclear	PRO	Unclear	Yes	FFPE	Yes	No	Yes	Yes	Yes	Yes	Unclear	N/A
Puertas Canteria 2012 ⁴⁵	Unclear	Unclear	Unclear	Unclear	No details	FFPE	Yes	No	Unclear	Unclear	No	No	No	N/A
Sato <i>et al</i> 2008 ⁴⁰	Unclear	Unclear	Unclear	Unclear	Yes	Snap frozen	Yes	No	Yes	Yes	Yes	Yes	Yes	BO segment length
Schulmann <i>et al</i> 2005 ³⁹	Unclear	Unclear	RET	Unclear	Yes	Snap frozen	Yes	No	Yes	Yes	Yes	Yes	No	N/A
Wang <i>et al</i> 2009 ³⁸	Yes	Unclear	PRO	RET	Yes	FFPE	Yes	No	Yes	No	Yes	Yes	No	N/A

BO, Barrett's oesophagus; FFPE, formalin-fixed paraffin-embedded.

are analysed with microarray technology, there will be an increasing number of negative results which are likely to be under-reported in the literature. As illustrated by Kyzas *et al* and Sekula *et al*,^{56 57} despite a large volume of literature, without standardisation of reporting and outcome measures, the research is often not suitable for translation into clinical use.

The variable quality of prognostic studies has been previously reported in systematic reviews^{58 59} and improvements suggested for collaboration and standardisation especially with regard to statistical analysis and outcome reporting.^{60–62} Poor quality of reporting was also a feature in this systematic review. For example, only 7 of 14 included studies documented both the number of patients and samples analysed in each group. Standardised reporting of epigenetic studies in BO should provide basic clinical details for each patient including age, sex, smoking status, BMI and previous OC diagnosis and treatment. Each patient used in the study should have temporal information regarding OGD and biopsies taken, the number of biopsies, BO length, BO islands, hiatus hernia, visible lesions and classification of visible lesions documented according to the British Society of Gastroenterology (BSG) minimum endoscopic reporting dataset.¹ In resection specimens, full histological reports should be available or at minimum staging tumour stage, nodal involvement, metastasis (TNM). It should be reported which patient samples have been used to perform epigenetic analysis and which methods of laboratory analysis have been used. Clear reporting of statistical analysis between cohorts must be provided to allow future meta-analysis of studies. Improved standardisation would make meta-analyses more feasible and lead to more informative systematic review results. This in turn would aid the translation of laboratory work into clinical trials.

CONCLUSION

The evidence from this systematic review is suggestive of a role for p16 as an individual epigenetic biomarker in predicting progression from BO to OADC. Prognostic models incorporating this and other markers also suggest a role for p16 in combination with HPP1, RUNX3 and/or clinical markers. Further large primary studies using current epigenetic techniques and standardised reporting are required to inform future models to further explore the role of epigenetics in progression to HGD and OADC.

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